A molecular update on pseudohypoaldosteronism type II

Ganesh Pathare, Joost G. J. Hoenderop, René J. M. Bindels, and Pedro San-Cristobal
Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Submitted 8 August 2013; accepted in final form 7 October 2013

The kidney plays an important long-term role in salt homeostasis and regulation of blood pressure (31). This is achieved by balancing ion reabsorption and secretion in the different segments of the nephron (20, 66). The aldosterone-sensitive distal nephron (ASDN) can be divided into distinct regions, namely 1) the distal convoluted tubule (DCT), 2) the connecting tubule (CNT), and 3) the cortical collecting duct (CCD). This is where the fine-tuning of sodium reabsorption and excretion in the kidneys occurs (2, 17, 90). In the DCT, the thiazide-sensitive Na-Cl cotransporter (NCC) reabsorbs ~10% of the filtered sodium and chloride from the pro-urine side (2, 23, 45, 58). About 2–3% of the filtered sodium is reabsorbed in the CNT by the epithelial sodium channel (ENaC). This transport reduces the luminal membrane potential in the pro-urine side and therefore sets up an ideal potential for potassium excretion through the renal outer medullary potassium channel (ROMK) channel in the DCT. This review focuses on new candidate genes such as KLHL3 and Cullin3, which are instrumental to unraveling novel signal transduction pathways involving NCC, to better understand the cause of PHAII along with the molecular mechanisms governing the pathophysiology of PHAII and its clinical manifestations.

PHA

PHA (75) (OMIM no. 145260) is a rare, heterogeneous, inherited syndrome (5, 16, 24, 64) characterized mainly by the decreased response to aldosterone (40). Symptoms arise as early as the prenatal period and/or early infancy and mainly consist of renal salt wasting with concomitant dehydration, hypotension, vomiting, and low weight gain. Laboratory findings include hyponatremia, hyperkalemia, and metabolic acidosis, due to increased sodium and decreased potassium excretion in the urine, high plasma renin levels, and high aldosterone levels in the plasma and urine (3, 4, 9), indicating the absence of a renoprotective response to mineralocorticoids (40, 57). Adrenal function and glomerular filtration rate (GFR) are normal. The gold standard for diagnosis is unresponsiveness to administration of exogenous mineralocorticoids (68, 69). This, together with high plasma concentrations of renin and aldosterone gives a definitive diagnosis of PHA. PHA is further divided into two types, autosomal dominant and autosomal recessive.

PHA type 2 (PHAII; OMIM no. 145260), also known as Gordon syndrome or familiar hyperkalemic hypertension (FHHt), is a rare heterogeneous syndrome inherited in an autosomal dominant manner (28, 29). PHAII is mainly characterized by...
hypertension, hyperkalemia, metabolic acidosis, diminished renal potassium excretion, hypercalciuria, and low plasma renin levels (47, 49). Serum aldosterone levels are either low or normal. Adrenal gland function and glomerular filtration rate (GFR) are reported as normal in most cases. Some patients with severe hypercalciuria can present with low bone mineral density, partly due to increased fractional excretion of calcium over several years (49). Symptoms related to the electrolyte disturbances and laboratory abnormalities can be detected as early as infancy (48), while hypertension may be detected two to four decades later. Over 50% of the patients affected with the disease are identified before the onset of blood pressure disturbances. Nonetheless, even with a timely diagnosis and appropriate treatment, all patients will eventually develop hypertension at some point during their adult life (20, 66). Patient lifestyle, gastronomic and cultural differences (mainly dietary salt intake), and mutations in relevant proteins could be reasons for the unpredictability of the onset of blood pressure disturbances in patients with PHAII (34, 37, 38, 84).

PHAII patients can be treated successfully with a chronic low-salt diet, or with thiazide-type diuretics, which are pharmacological inhibitors of NCC (47). Thiazide diuretics are an established treatment for correcting the clinical and laboratory manifestations of PHAII (47, 49). Despite the clear role of NCC as one of the main candidates in the onset of PHAII, genomic linkage analysis demonstrated no significant association of mutations in the SLC12A3 gene on chromosome 16 in patients with PHAII (14). This observation raised doubts that PHAII is caused by mutations that activate NCC. However, intravenous infusion of sodium sulfate increases renal clearance of potassium and simultaneously reduces serum potassium levels (47, 49). Serum aldosterone levels are either low or normal in PHAII patients (34, 37, 38, 84).

Pathophysiology of PHAII

Although it is clear that hypertension in PHAII patients is a result of increased Na+ reabsorption via NCC, there are various mechanisms through which this can occur (Fig. 1). Mutations in the genes encoding WNK kinases (WNK1 and WNK4) were identified in 2001 as causative factors for PHAII (81). Recent animal studies suggest that WNK4 is upregulated in PHAII and loss of WNK4 results in a Gitelman-like syndrome (8). Mutations that cause charge-changing amino acid substitutions within the kinase motif of WNK4 also abolish the inhibitory effect on NCC and result in PHAII (81). Similarly, it is interesting to note that wild-type WNK4 inhibits NCC and mutated WNK4 stimulates NCC activity (59, 81). In short, altered WNK expressions (particularly WNK4), whether due to mutations within the WNKs or the molecules that control WNKs, are the main culprit in the etiology of PHAII.

In PHAII, phosphorylation of NCC is always increased, suggesting that the Ste20-related proline alanine-rich kinase (SPAK)/oxidative stress-responsive kinase (OSR) 1 pathway is activated by WNK4 and may be ubiquitinated by KLHL3/Cullin3. Nedd4-2 is known to modulate NCC via the aldosterone- serum- and glucocorticoid-regulated kinase 1 (SGK1)-Nedd4-2 pathway. At the same time, at least in vitro, the renal outer medullary potassium (ROMK) channel is inhibited by WNK1–3. Along the DCT, NCC retrieval from the plasma membrane is inhibited by WNK4. The left (yellow) column represents the pro-urine, and the right (red) column represents blood. O. transporter; = ion channel. Arrows indicate upregulation, and cut lines indicate inhibition. Fine lines indicate established pathways, while dotted lines indicate possible pathways that may be valid at least in in vitro models. TRPV5, transient receptor potential V5 channel.
The pathophysiology of hypertension in PHAII includes increased cell surface expression and/or activity of NCC, resulting in enhanced reabsorption of sodium and chloride. These changes in NCC arise in part due to mutations in WNK1 and WNK4, but it is likely that there are additional mutations that have not yet been described. Enhanced sodium reabsorption results in intercellular volume expansion and inhibits renin secretion, resulting in low plasma renin activity in all patients. Plasma aldosterone levels are variable, but are relatively low given the range of hyperkalemia (82).

**Main Players in Pathophysiology of PHAII**

WNKs. The WNKs are a small family of serine/threonine kinases, so called because they lack a lysine residue in the kinase domain (lysine = K) (81). The role of the WNK family in the maintenance of normotensive blood pressure has been well established (Fig. 2). There has been interest in parsing the activity of the individual WNK isoforms upon activation by aldosterone. Serum- and glucocorticoid-regulated kinase 1 (SGK1), which regulates ion channel levels at the cell surface, is activated by aldosterone and WNK4 (32). WNKs share ~40% sequence identity overall (14% in the amino-terminal domain, 85% in the kinase domain, and 17% in the carboxy-terminal domain). The conserved domains in their carboxy-terminal regions are important for the protein-protein interactions required to regulate ion balance, cell proliferation, cell volume regulation, cell survival, cell signaling, and organ development. The expression of WNK1 is widespread throughout most of the body (11, 72, 85), while WNK4 expression presents a more limited expression pattern. WNK1 has two isoforms. The L-WNK1 is the long isoform of WNK1 and is expressed ubiquitously. The other isoform, called KS-WNK1, is only present in the kidney (72). The L-WNK1 isoform enhances Na⁺ reabsorption in the kidney and decreases K⁺ secretion, while KS-WNK1 has the opposite effects (79). WNK4 has been reported in the brain, heart, lung, kidney, testis, epididymis, liver, pancreas, colon, and prostate (36). These tissues are rich in epithelia involved in ion transport (67). In the kidneys, WNK1 and WNK4 expression have been reported primarily in DCT, CNT and CD (36, 54, 81). WNK1 is localized in the cytoplasm of epithelial cells of the DCT and CD. WNK1 expression is highest in the DCT, while sharply decreasing in the CD (54, 55). However, WNK4 is localized in the tight junctions and cytoplasm of the DCT and CD. WNK4 is also expressed in the thick ascending limb of Henle’s loop (TAL) (11, 36).

The main function of WNKs in the distal part of the nephron is regulating ion handling for maintaining electrolyte balance. Many studies have been conducted in heterologous expression systems such as *Xenopus laevis* oocytes, cell lines, and transgenic animals, to fully elucidate WNK1 and WNK4 function. The current knowledge shows the myriad ways by which WNK regulates NCC activity: how it controls the amount of NCC at the cell plasma membrane (38, 83, 86, 88), regulates degradation via the lysosomal pathway (7, 26, 72, 92), and directs phosphorylation in the presence of hormones such as angiotensin II (67, 91). Additionally, there is evidence showing that WNK1 phosphatidyl cholin 4 and increases paracellular chloride permeability (60). WNK1 also enhances ENaC activity by indirectly inducing SGK1 phosphorylation. SGK1, in turn, phosphorylates and inhibits Nedd4–2. This inhibits clathrin-mediated endocytosis of ENaC and, therefore, increases ENaC levels at the cell membrane (30). WNK1 also activates SPAK and OSR1, which increases NKCC1 and NKCC2 activity and reduce the activity of the K⁺/2Cl⁻ co-transporter 3 (KCC3) (50). It is also known to inhibit calcium entry through the TRPV4 channel by decreasing the expression of TRPV4 on the cell surface (19). WNK1 has two isoforms. The L-WNK1 is the long isoform of WNK1 and is expressed ubiquitously in all cells throughout the body. The other isoform is called KS-WNK1 and is only present in the kidney (72). The L-WNK1 isoform enhances sodium reabsorption in the kidney and decreases potassium secretion, while KS-WNK1 has opposite effects (79).

Recent reports indicate that WNK4 has a major role in controlling NCC activity and is a critical player in the pathophysiology of PHAII. Several studies done in oocytes, transgenic mice, and human embryonic kidney 293 (HEK293) cells have shown that WNK4 is a negative modulator of NCC activity until its primary structure is changed, upon which it becomes an activator of NCC (22). Tacrolimus upregulates NCC activity in parallel with WNK4 (33), while norepinephrine upregulates NCC with downregulation of WNK4 (51). Recent updates show that NCC phosphorylation in the kidney is highly dependent on WNK4 (8). WNK4 modulates ROMK via WNK1 and WNK4 and the endocytic scaffold protein intersectin (52), forming two complexes consisting of ROMK interacting with WNK4 and WNK1-WNK4 interacting with intersectin (53).
There are interesting findings from transgenic mice with respect to WNKs. Lalioti et al. (42) developed and studied WNK4 transgenic mice (Q562E) that display a phenotype closely resembling PHAII. They showed that mice overexpressing WNK4 with the PHAII mutation display hypertension, hyperkalemia, metabolic acidosis, and hypercalcemia. Interestingly, this phenotype was reversed by backcrossing these mice with NCC knockout mice, clearly indicating that increased NCC activity is a hallmark of the PHAII phenotype (42). In another study, Yang et al. (88) analyzed a second mouse model with one normal copy and one mutant copy of WNK4 (D561A). WNK4 knockin mice with a PHAII mutation show increased expression and phosphorylation of NCC. As expected, this mouse model exhibited hypertension and hyperkalemia, which was corrected by thiazide treatment. Urinary potassium excretion was also enhanced with sodium sulfate, although there was no increase in overall levels of ROMK (88).

Recently, there have been ground-breaking discoveries in the field of WNK4 using animal models. The report published by Wakabayashi et al. (80) demonstrated that WNK4 protein levels are rather increased in WNK4 knockin (D561A) mice, which was not reported in the previous article by Yang et al. (88). In 2012, Gamba et al. (8) analyzed total WNK4 knockout mice and found that total loss of WNK4 in mice leads to a Gitelman-like phenotype with mild hypokalemia, metabolic alkalosis, hypocloremia, hypomagnesemia without hypocalciuria, and hypotension. Mice heterozygous for WNK1 survive to adulthood; their blood pressure was moderately decreased, but they have no detectable abnormalities in levels of electrolytes or biochemical parameters in the blood and urine (89). Recently, Vidal-Petiot et al. (76) presented a simple and clear message conceived from phenotyping of WNK1+/−/FHH mouse model. The large deletions in the first intron of the WNK1 gene in the mouse increased the expression of long WNK1 (L-WNK1), which leads to the classic symptoms of FHH (76). The unaltered activity of ENaC and decreased ROMK expression in the WNK1+/−/FHH mouse model are other hallmark findings of this study.

**SPAK and OSR1.** SPAK and OSR1 are two serine/threonine kinases belonging to the kinase subfamily VI. They have similar structures, sharing homology in their N-terminal catalytic domain and in two conserved regions known as the S-motif (70%) and the conserved C-terminal domain (80%) (77, 78). SPAK and OSR1 are downstream regulators of WNKs, and they are present in several tissues, including the kidney. However, their expression is distinct: in the kidney, SPAK is present in the TAL and DCT (62). On the other hand, NCC and NKCC2 are only present in the kidney, and in fact they colocalize to the same nephron segments as SPAK itself (62). Phosphorylation of NCC and NKCC2 by SPAK/OSR1 has been highlighted by studying the phenotype of OSR1−/− and SPAK-deficient mouse models (62). At least one of the intronic SNPs in STK39 (gene coding SPAK) appeared to increase SPAK expression, which could enhance NCC phosphorylation and hence salt retention and blood pressure. More recent studies demonstrated that SPAK and OSR1 kinases phosphorylate human NCC at three conserved residues (Thr46, Thr55, and Thr60) (63). In vitro studies have shown that SPAK and OSR1 directly phosphorylate Thr203, Thr207, and Thr212 residues present at the N-terminal region of the human NKCC1 (78). Knocking down SPAK leads to Gitelman syndrome. Furthermore, SPAK has a major role in NKCC1 action in the vascular smooth muscle (12, 87). Mouse protein-25 (MO25) is an emerging molecule characterized as a master regulator of SPAK/OSR1 (18). Interestingly, MO25 isoforms strongly activate SPAK/OSR1 and therefore dramatically enhance their ability to phosphorylate cotransporters like NCC and NKCC2.

Different hormones have the ability to manipulate NCC expression and activity. It is clear that aldosterone, insulin, and vasopressin exert similar stimulatory effects on NCC (61). Similarly, angiotensin II has a proven role in activating NCC (67). As SPAK and OSR1 are WNK targets, it is interesting to speculate on the role of these hormones in a WNK/SPAK/OSR1/NCC signaling cascade. A recent publication from the Uchida group (10) confirms that phosphorylation of NCC in the kidney is totally governed by the WNK-OSR1/SPAK signaling pathway. Moreover, the PHAII phenotype caused by the WNK4 mutant is also controlled by this signal cascade (10). Hence the most recent data indicate SPAK/OSR1 are the ultimate candidates responsible for the PHAII phenotype, and inhibition of these kinases would be key to manage high blood pressure in PHAII.

**KLHL3 and Cullin3.** The role of WNKs in protein ubiquitination in PHAII has gathered considerable attention because of the discovery of two unique kinases KLHL3 and Cullin3 (25, 80). Impaired function of Cullin3 in humans has been linked to various anomalies such as metabolic disorders, muscular, and neuronal degeneration (25). In 2012, Jeunemaitre et al. (46) identified KLHL3 as a new gene responsible for PHAII, and

![Fig. 3. Molecular adaptations in PHAII. Enhanced NCC activity causes increased sodium reabsorption in the DCT, which leads to decreased sodium and calcium reabsorption in the proximal tubule and decreased potassium excretion in DCT and the connecting tubule (CNT). These events are the main framework for the PHAII, i.e., high blood pressure, hyperkalemia, metabolic acidosis, and osteoporosis. Decreased ROMK activity reduces potassium excretion and causes hyperkalemia. Mutations in WNK4 downregulate TRPV5 and decrease calcium reabsorption in the DCT, causing urinary calcium loss and osteoporosis.](http://ajprenal.physiology.org/)

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hence it could have a role in regulating NCC. Moreover, they showed that this particular gene is expressed in the DCT where it controls sodium reabsorption, which can be corrected with thiazide diuretics (46). Thus KLHL3 colocalizes with NCC where it regulates NCC expression at the cell surface. Boyden et al. (6) identified another gene named Cullin3 as a new member of the complex signaling pathway regulating ion homeostasis in the DCT. They found that Cullin3 mutations are dominant and de novo, while KLHL3 mutations could be recessive or dominant. The study also illustrated that KLHL3 is related to the BTB-BACK-kelch family that ligates substrates for Cullin3-based ubiquitination. These two studies clearly establish a role for KLHL3 and Cullin3 as participants in the complex signaling pathway regulating sodium reabsorption, but there is further study needed to unravel the mechanism of sodium handling by these two genes.

Recent publications have unraveled the role of KLHL3-Cullin3-WNK interaction and its role in the pathophysiology of hypertension. Takahashi et al. (73) reported that KLHL2 along with KLHL3 can be coimmunoprecipitated with all four WNK isoforms. The KLHL2-Cullin2 decreased the expression of WNK1, WNK3, and WNK4 in vitro by ubiquitination. (73). Another elaborative study done by Shibata et al. (70) showed that mutations in both KLHL3 and WNK4 impair WNK4 binding, ubiquitination, and degradation and, therefore, PHAII-causing mutations markedly increase the WNK4 expression in kidney in vivo. On the other hand, Ohta et al. (59) reported that mutations in WNK4 disable its normal interaction with KLHL3 and further suggest that the CUL3-KLHL3 ligase complex regulates blood pressure through its ability to interact with and ubiquitinate WNK isoforms (59). Recently, Wakabayashi et al. (80) showed that KLHL3 interacts with Cullin3 and WNK4, which induces ubiquitination of WNK4 and ultimately downregulates WNK4. PHAII-causing mutations attenuate these interactions and result in reduced ubiquitination of WNK4, which could be responsible for the hypertension phenotype. It can also be speculated that KLHL3 participates in NCC trafficking through its association with the actin cytoskeleton via its C-terminal Kelch domain (71). However, it is now relevant to study these genes in animal models to analyze the conventional blood pressure phenotype.

Nedd4-2. Nedd4-2 is an ubiquitin ligase and is mainly responsible for endocytosis and lysosomal degradation. The role of Nedd4-2 as a regulator of ENaC and other ion channels has recently been a subject of considerable interest. After Nedd4-2 binding, ENaC undergoes ubiquitination and is subsequently targeted for internalization. This mechanism indicates that dysfunction of Nedd4-2 is more related to the pathophysiology of Liddle’s syndrome than PHAII. However, some recent findings support a role in the pathophysiology of PHAII. Aldosterone is known to increase ENaC expression by promoting SGK1-mediated phosphorylation of ubiquitin-protein ligase Nedd4-2. Interestingly, aldosterone also activates NCC (39). The Staub group (1) demonstrated that Nedd4-2 coimmunoprecipitates with NCC, and enhances NCC ubiquitination in a X. laevis model (1). They proposed that Nedd4-2 but not its inactive mutant decreases NCC activity and surface expression. Ronzaud et al. (65) confirmed, in a mouse model, that Nedd4-2 deficiency leads to hypertension through NCC-mediated pathways. However, the particular phenotype observed did not display hyperkalemia, as ROMK levels were increased in the kidneys of Nedd4L knockout animals. As the research connecting SGK1 and Nedd4-2 is still in its nascent stages, further work is necessary to understand the mechanism.

Conclusion

PHAII is a rare heterogeneous syndrome involving the kidneys and vasculature. The symptoms include hypertension, hyperkalemia, and metabolic acidosis. Researchers have described the remarkable efficacy of thiazide diuretics for the treatment of hypertension and other biochemical abnormalities seen in PHAII patients (Fig. 3). This observation led to the conclusion that PHAII is caused by gain-of-function mutations in NCC. In the last 10 years, several research groups have identified novel proteins involved in the pathogenesis of PHAII, namely, WNK1, WNK4, OSR1, SPAK, KLHL3, Cullin3, and Nedd4-2. The expression of these proteins in the kidney is limited to the DCT and CD. Although PHAII is a rare disorder, unraveling the underlying mechanisms of PHAII would contribute to a better understanding of the function of the distal part of the nephron and of the pathophysiology of renal diseases. Additional insights are expected from future studies, using exome sequencing from patients, in vitro models, and transgenic animal models.

GRANTS

P. San-Cristobal is a fellow of the European Society of Hypertension (ESH) and financially supported by the Netherlands Organization for Scientific Research (VENI ZonMw 916.12.046). In addition, we acknowledge the financial support of the Dutch Kidney foundation (PhD 12.14) and the European Union 7TH Framework Programme EURenOMICS (FP7/2007–2013, agreement no. 305608).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: G.P. and P.S.-C. prepared figures; G.P. and P.S.-C. drafted manuscript; G.P., J.G.H., R.J.B., and P.S.-C. edited and revised manuscript; G.P., J.G.H., R.J.B., and P.S.-C. approved final version of manuscript; R.J.B. interpreted results of experiments.

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